

## DESCRIPTION

### METHOD OF PRODUCING VIRUS

### TECHNICAL FIELD

The present invention relates to a method of producing a virus.

### BACKGROUND ART

Recently, a risk of zoonotic infectious disease such as BSE (Bovine Spongiform Encephalopathy) infection and Avian Influenza virus infection, has started to be a concern. Moreover, products using ingredients or materials primarily originated from animals (such as vaccines, blood preparations, cell culture/genetic recombinant preparations, and cellular tissue medical products) have problems of a high risk where an infectious agent is mixed thereinto, and an undeniable likelihood of containing an unknown infectious agent, as well as a limitation in the inactivation treatment of infectious agents, and the like. As a countermeasure against such problems, legal countermeasures for safety have been enhanced, such as newly providing a framework for "bio-originated products" by the amendment of Japanese Pharmaceutical Law in 2003, and there has become a desire for the development of medical products free from animal-origin components.

Many attempts have been made for obtaining a culture condition not requiring a serum, such as bringing serum-free mediums free from animal-origin components into the market by respective medium manufacturers. However, in a culture method using a serum-free medium and a microcarrier where the surface of the carrier is electrically charged at an appropriate amount so as to adhere cells, as a support for culturing adhesive cells, there has been a problem in that the attachment rate to the microcarrier is reduced, making it difficult to efficiently culture a large amount of cells. As a result, conventionally, under such a culture condition, there has been used a carrier containing animal-origin components such as a microcarrier coated with denatured pig collagen, as described for example in Non Patent Document 1.

Moreover, there is disclosed in Patent Document 1, a bead for culturing animal cells which has a high cell-adhering property and a high cell-proliferating property, and, even in a serum-free culture medium, gives a cell-adhering property and a cell-proliferating property

which are equivalent or more to those in serum-containing medium. However, although Patent Document 1 shows that cells can be efficiently cultured under a serum-free condition in a cell culture, there is no disclosure of a condition for producing a virus such as a virus inoculation or proliferation method.

Moreover, there is disclosed in Patent Document 2, a method of producing a virus including steps of: obtaining a vertebrate cell culture such as Vero cells; proliferating the cells only in a protein-free medium (free from serum or non-serum protein); infecting this culture with a virus; incubating the virus-infected cell culture; proliferating the virus in the medium; and producing the virus-containing medium. Furthermore, there is disclosed a usage of a protease originated from a procaryote supply source as a substance which enhances the virus activity. Patent Document 2 describes that, according to this method, the obtained virus do not contain various impurity compounds originated from a human or animal supply source, nor a protein serving as a pathogenic substance. However, in an Example a trypsin which is an animal-origin component is used as a substance which enhances the virus activity.

Furthermore, there is disclosed in Patent Document 3, a method of producing a virus infected insect cell not using a naturally-originated protein but using a cell-adhesive support having a high cell-adhering property. This production method is a method of producing a virus infected insect cell, comprising steps of; using a cell-adhesive artificial peptide and/or a cell-adhesive auxiliary artificial peptide to adhere a poikilothermic animal-origin cell and a substrate, and using this cell-adhered substrate for culturing cells. Patent Document 3 describes that, by not using a naturally-originated protein for a substrate, there is no risk of containing an infectious substance such as a human-infective virus, and the safety is high. However, there is no disclosure of a cell dispersing agent free from animal-origin components or an adhesive cell originated from a homoiothermic animal. On the other hand, for the subculture of cells in Non Patent Document 1, an animal-origin protease (such as pig-origin trypsin) is used as a cell dispersing agent.

Patent Document 1: Japanese Unexamined Patent Publication No. 2003-189848

Patent Document 2: Japanese Patent Publication No. 3158157 (WO96/15231 pamphlet)

Patent Document 3: Japanese Unexamined Patent Publication No. 2003-210166

Non Patent Document 1: Otfried Kistner et al. "Development of a Novel Mammalian Cell (Vero) Derived influenza Vaccine" Poster Presented at: Options for Control Of Influenza V, Okinawa, Japan, October 7-11, 2003

## DISCLOSURE OF THE INVENTION

### Problems to be solved by the Invention

Therefore, an object of the present invention is to provide a safe and efficient method of producing a virus which is free from animal-origin components in the whole process from culturing adhesive cells to producing the virus on an industrial scale by the cell culture.

### Means for solving the Problem

The above problems have been considered and earnestly studied, resulting in a finding of a safe and efficient method of producing a virus by using a culture material which is free from animal-origin components, and thus conceiving the present invention.

The scope of the method of producing a virus of the present invention comprises the points of: adhering adhesive cells to a support which has a polypeptide (P) having at least one cell-adhesive minimum amino acid sequence (X) per molecule, and is free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components; and then inoculating and proliferating a virus in the cells obtained by culturing the adhesive cells.

### Effects of the Invention

In the method of producing a virus of the present invention, the virus can be safely and efficiently produced. Therefore, the method of the present invention is suitable for producing a vaccine.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing changes in cell density with time in Example 1 and Comparative Examples 1 to 3.

FIG. 2 is a graph showing changes in virus production quantity by ELISA with time in Example 1 and Comparative Examples 1 to 3.

FIG. 3 is a graph showing changes in virus production quantity by HA with time in Example 1 and Comparative Examples 1 to 3.

## BEST MODE FOR CARRYING OUT THE INVENTION

In the present invention, “free from animal-origin components” means free from components originated from homoiothermic animals, in particular, animals such as mammals (for example, human, cattle, pig, dog, rabbit, cat, and the like), birds, and fishes.

Moreover, in the present invention, an adhesive cell is not specifically limited as long as it is a homoiothermic animal-origin cell which grows by adhering onto a solid surface and is capable of proliferating a virus. Examples thereof include an epithelial cell (such as Vero, MDCK, CHO, HEK293, and COS), a tumor cell (such as Hela and VACO), an endothelial cell (such as HUVEC and DBAE), a leukocyte (such as HIT-T15), a fibroblast (such as WI38, BHK21, and SFME), a muscle cell (such as HL1 and C2C12), a nerve/endocrine cell (such as ROC-1 and IMR-32), and a primary cell (such as primary chick embryo, primary quail embryo, and primary rabbit kidney). Among these, preferred are an epithelial cell and a primary cell, more preferably Vero, MDCK, and a primary cell, particularly preferably Vero and MDCK, and most preferably Vero.

Moreover, examples of the virus to be inoculated in the adhesive cells in the present invention include Flaviviridae (such as a Yellow fever virus, a Japanese encephalitis virus, a hepatitis C virus, and a Dengue fever virus), Orthomyxoviridae (such as an Influenza virus), Adenoviridae (such as a Human adenovirus 1-34), Herpesviridae (such as a Herpes simplex I, a Herpes simplex II, a Pseudorabies virus, a Varicella-zoster virus, and a Human cytomegalovirus), Picornaviridae (such as an Aphthovirus O, a Foot-and-mouth disease virus, a Poliovirus, and a Hepatitis A virus), Paramyxoviridae (such as a Measles virus, a Mumps virus, and a Sendai virus), Togaviridae (such as a Rubella virus and an Encephalitis virus), Poxviridae (such as a Vaccinia virus, a Variola virus, a Cowpox virus, and a Monkeypox virus), Retroviridae (such as a Human immunodeficiency virus (HIV) and a Human T-lymphotropic virus), and Coronaviridae (such as an Avian infections bronchitis virus) (Dictionary of biochemistry (second edition), published by Tokyo Kagaku Dojin (11 September 1997)). Among these, preferred are viruses belonging to Flaviviridae, Orthomyxoviridae, Adenoviridae, Herpesviridae, Picornaviridae, Paramyxoviridae, Togaviridae, or Poxviridae, more preferred are viruses belonging to Flaviviridae, Orthomyxoviridae, Paramyxoviridae, or Togaviridae, particularly preferred are viruses belonging to Orthomyxoviridae, Paramyxoviridae, or Togaviridae, and most preferred are viruses belonging to Orthomyxoviridae.

Furthermore, the culture material (such as a medium, a cell dispersing agent, and a support for adhering adhesive cells) free from animal-origin components used in the present invention is free from animal-origin components, and therefore has advantages in that: it can

minimizes a likelihood of contamination due to foreign substances; it is free from unknown infectious agents; and it has a low risk of being mixed with infectious agents, and thus there is no need for performing a treatment for deactivating the infectious agents. Hereunder is a detailed description of the culture material free from animal-origin components.

The medium used for culturing an adhesive cell or producing a virus is not specifically limited as long as it is a medium free from a serum or a protein being animal-origin components (hereunder, serum-free medium). Examples thereof include a commercially available serum-free medium {such as OPTIPRO<sup>TM</sup>SFM medium (manufactured by Invitrogen), VP-SFM (manufactured by Invitrogen), and EX-CELL 525 (manufactured by JRH Bioscience)}, a basal medium (such as Eagle MEM medium, Dulbecco's Modified Eagle medium, Iscove's medium, RPMI1640 medium, Ham F10 medium, Ham F12 medium, MCDB 105 medium, MCDB 107 medium, MCDB 110 medium, MCDB 131 medium, MCDB 151 medium, MCDB 152 medium, MCDB 153 medium, MCDB 201 medium, MCDB 302 medium, and MEDIUM 199) and the mixed medium thereof. Among these, from the viewpoints of medium preparation etc., preferred is a commercially available serum-free medium, more preferred are OPTIPRO<sup>TM</sup>SFM medium, VP-SFM, and EX-CELL 525, particularly preferred are VP-SFM and EX-CELL 525, and yet more particularly preferred are VP-SFM. This VP-SFM is suitable for culturing a cell system, such as Vero, COS-7, MDCK, BHK-21, and HEP-2, that is used for proliferation of a virus. The serum-free medium may be appropriately added with, as a non animal-origin additive, a hormone (such as insulin and hydrocortisone) originated from genetic recombinant bacteria or the like, a cell growth factor (such as an epidermal growth factor (EGF), a platelet-derived growth factor (PDGF), and a fibroblast growth factor (FGF)), and an antimicrobial (such as kanamycin), so as to stably improve the cell-proliferating property.

The material of the support is not specifically limited as long as it is a material capable of adhering an adhesive cell. However, from the viewpoints of cytotoxicity etc., it preferably has the following materials as a primary component.

- (1) Synthetic polymer: vinyl resin, polyester, polyurethane, epoxy resin, nylon, polycarbonate, and the like.
- (2) Natural polymer: cellulose, cellulose derivative (such as cellulose diacetate and cellulose triacetate), dextran, and the like.
- (3) Inorganic substance: aluminum oxide, hydroxyapatite, titanium oxide, silica, glass, and the like.



Among these, preferred are a synthetic polymer, a natural polymer, and hydroxyapatite, more preferred are a synthetic polymer and a natural polymer, particularly preferred is a synthetic polymer, and most preferred are a vinyl resin and nylon.

Examples of the vinyl resin include a vinyl monomer (such as an acrylic monomer, alkene, and styrene), and a polymer comprising polyfunctional monomers and the like as a constitutional unit as necessary (such as polystyrene, crosslinked polystyrene, polymethyl(metha)acrylate, poly(metha)acrylamide, crosslinked polyacrylamide (such as a copolymer of acrylamide and ethylene glycol diacrylate), and poly(metha)acrylonitrile). Among these, preferred is a resin comprising styrene as an essential constitutional unit, and more preferred is crosslinked polystyrene.

Examples of the polyfunctional monomer include divinylbenzene, ethylene glycol di(metha)acrylate, trivinylbenzene, and trimethylolpropane tri(metha)acrylate.

The shape of the support is not specifically limited as long as it is a shape capable of adhering an adhesive cell, and may be any one of a plate, a petri dish, a T-flask, a roller bottle, a microcarrier, a hollow fiber, a sheet (such as a film, a foam (sponge), and a cloth), and a gel. Among these, from the viewpoints of the culture volume etc., preferred are a petri dish, a T-flask, a roller bottle, a microcarrier, and a hollow fiber, more preferred are a roller bottle, a microcarrier, and a hollow fiber, particularly preferred are a microcarrier and a hollow fiber, and most preferred is a microcarrier.

The form of the microcarrier includes a solid type and a porous type, any one of which may be used. However, a solid type is preferred, from the viewpoints of the efficiency of supplying nutrients and oxygen to the cells, the recovery rate of the cells, etc. Moreover, as to the shape of the microcarrier, either globular or flat (oval) may be used.

In the case of the solid type, the particle diameter ( $\mu\text{m}$ ) of the microcarrier is preferably 20 to 2000, more preferably 40 to 1000, and particularly preferably 80 to 500. On the other hand, in the case of the porous type, the particle diameter ( $\mu\text{m}$ ) is preferably 30 to 25000, more preferably 60 to 12000, and particularly preferably 120 to 6000. Within this range, the cell growth is further increased.

The true specific gravity of the microcarrier is not specifically limited. However, in a general method of culturing while stirring a microcarrier together with a medium, preferably beads are floating during the stirring, and they are sedimented when the stirring is stopped. From such a viewpoint, the true specific gravity ( $\text{g}/\text{cm}^3$ ) of the microcarrier is preferably 1.00 to 1.10, more preferably 1.01 to 1.08, and particularly preferably 1.01 to 1.05.

The microcarrier can be readily commercially available, and the products as follows may be used.

- (1) Made from polystyrene: Biosilon (manufactured by Nalge Nunc International), Plastic beads (manufactured by Solohill Engineering), Cytosphere (manufactured by Lux), and the like.
- (2) Made from polyacrylamide: Biocarrier (manufactured by Bio-Rad Laboratories), and the like.
- (3) Made from polyurethane: PUF (manufactured by INOAC), and the like.
- (4) Made from cellulose: Cellsnow (manufactured by Biomaterial), and the like.
- (5) Made from dextran: Cytodex (manufactured by Amersham Pharmacia), and the like.
- (6) Made from glass: SIRAN (manufactured by Scott Medical Products), and the like.

The support contains a polypeptide (P) having at least one cell-adhesive minimum amino acid sequence (X) per molecule. By containing the polypeptide (P), a highly efficient production of a virus can be realized without using an animal-origin material.

The “cell-adhesive minimum amino acid sequence” means a minimum amino acid sequence having a property of being recognized by an integrin receptor of a cell, and thus facilitating adhering of the cell to a substrate.

From the viewpoints of cell-adhering property etc., the number of cell-adhesive minimum amino acid sequences (X) contained in the polypeptide (P) is preferably 1 to 50 per molecule (P), more preferably 3 to 30, and particularly preferably 4 to 20.

As for the cell-adhesive minimum amino acid sequence (X), any sequence may be used as long as it acts as an adhesive signal, and there may be used those described in “Pathophysiology” Vol.9, No.7, 1990, p527, published by Nagai Shoten Co., Ltd. Among these, from the viewpoints of abundant types of cells that are readily adhered, etc., preferred are an Arg Gly Asp sequence, a Leu Asp Val sequence, a Leu Arg Glu sequence, a His Ala Val sequence, and sequences represented by SEQ ID NOs:1 to 8, more preferred are an Arg Gly Asp sequence, a His Ala Val sequence, and a sequence represented by SEQ ID NO:7, and particularly preferred are an Arg Gly Asp sequence.

From the viewpoints of improving the thermal stability of (P) etc., the polypeptide (P) preferably has an auxiliary amino acid sequence (Y) in addition to the cell-adhesive minimum amino acid sequence (X).

As for the auxiliary amino acid sequence (Y), there may be used an amino acid sequence other than the minimum amino acid sequence (X). From the viewpoints of improving

the thermal resistance of the polypeptide (P) etc., it is preferably a sequence having Gly and/or Ala.

Examples of the auxiliary amino acid sequence (Y) include sequences having a (Gly Ala)<sup>a</sup> sequence, a (Gly Ala Gly Ala Gly Ser)<sup>b</sup> sequence, a (Gly Ala Gly Ala Gly Tyr)<sup>c</sup> sequence, a (Gly Ala Gly Val Gly Tyr)<sup>d</sup> sequence, a (Gly Ala Gly Tyr Gly Val)<sup>e</sup> sequence, an (Asp Gly Gly (Ala)<sup>f</sup> Gly Gly Ala)<sup>g</sup> sequence, a (Gly Val Pro Gly Val)<sup>h</sup> sequence, a (Gly)<sup>i</sup> sequence, an (Ala)<sup>j</sup> sequence, a (Gly Gly Ala)<sup>k</sup> sequence, a (Gly Val Gly Val Pro)<sup>m</sup> sequence, a (Gly Pro Pro)<sup>n</sup> sequence, a (Gly Ala Gln Gly Pro Ala Gly Pro Gly)<sup>o</sup> sequence, a (Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly Ala Pro Gly Leu Gln)<sup>p</sup> sequence, and/or a (Gly Ala Pro Gly Thr Pro Gly Pro Gln Gly Leu Pro Gly Ser Pro)<sup>q</sup> sequence. Among these, preferred are sequences having a (Gly Ala)<sup>a</sup> sequence, a (Gly Ala Gly Ala Gly Ser)<sup>b</sup> sequence, a (Gly Ala Gly Ala Gly Tyr)<sup>c</sup> sequence, a (Gly Ala Gly Val Gly Tyr)<sup>d</sup> sequence, a (Gly Ala Gly Tyr Gly Val)<sup>e</sup>, a (Asp Gly Gly (Ala)<sup>f</sup> Gly Gly Ala)<sup>g</sup> sequence, a (Gly Val Pro Gly Val)<sup>h</sup> sequence, a (Gly Val Gly Val Pro)<sup>m</sup> sequence, and/or a (Gly Pro Pro)<sup>n</sup> sequence, more preferred are sequences having a (Gly Ala Gly Ala Gly Ser)<sup>b</sup> sequence, a (Gly Val Pro Gly Val)<sup>h</sup> sequence, a (Gly Val Gly Val Pro)<sup>m</sup> sequence, and/or a (Gly Pro Pro)<sup>n</sup> sequence, and particularly preferred are sequences having a (Gly Ala Gly Ala Gly Ser)<sup>b</sup> sequence.

Here, a is an integer of 5 to 100, b, c, d and e are integers of 2 to 33, f is an integer of 1 to 194, g is an integer of {1} to {200/(6+f)} that has been truncated to omit fractions, h is an integer of 2 to 40, i and j are integers of 10 to 200, k is an integer of 3 to 66, m is an integer of 2 to 40, n is an integer of 3 to 66, o is an integer of 1 to 22, and p and q are integers of 1 to 13.

The auxiliary amino acid sequence (Y) preferably contains glycine (Gly) and/or alanine (Ala). If glycine (Gly) and alanine (Ala) are contained, the proportion of total content thereof (%) is preferably 10 to 100 based on the total number of amino acids of the auxiliary amino acid sequence (Y), more preferably 20 to 95, particularly preferably 30 to 90, and most preferably 40 to 85. Within this range, the thermal resistance is further improved.

If both of glycine (Gly) and alanine (Ala) are contained, their content number ratio (Gly/Ala) is preferably 0.03 to 40, more preferably 0.08 to 13, and particularly preferably 0.2 to 5. Within this range, the thermal resistance is further improved.

From the viewpoints of improving the thermal resistance etc., the number of the auxiliary amino acid sequences (Y) contained in the polypeptide (P) is preferably 2 to 51 per molecule (P), more preferably 3 to 35, and particularly preferably 4 to 20. Moreover, the polypeptide (P) may contain a plurality of types of auxiliary amino acid sequences (Y).



The auxiliary amino acid sequence having a (Gly Ala)<sup>a</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 9 to 11.

The auxiliary amino acid sequence having a (Gly Ala Gly Ala Gly Ser)<sup>b</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 12 to 14.

The auxiliary amino acid sequence having a (Gly Ala Gly Ala Gly Tyr)<sup>c</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 15 to 17.

The auxiliary amino acid sequence having a (Gly Ala Gly Val Gly Tyr)<sup>d</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 18 to 20.

The auxiliary amino acid sequence having a (Gly Ala Gly Tyr Gly Val)<sup>e</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 21 to 23.

The auxiliary amino acid sequence having an (Asp Gly Gly (Ala)<sup>f</sup> Gly Gly Ala)<sup>g</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 24 to 26.

The auxiliary amino acid sequence having a (Gly Val Pro Gly Val)<sup>h</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 27 to 30.

The auxiliary amino acid sequence having a (Gly)<sup>i</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 31 to 33.

The auxiliary amino acid sequence having an (Ala)<sup>j</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 34 to 36.

The auxiliary amino acid sequence having a (Gly Gly Ala)<sup>k</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 37 to 39.

The auxiliary amino acid sequence having a (Gly Val Gly Val Pro)<sup>m</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 40 to 42.

The auxiliary amino acid sequence having a (Gly Pro Pro)<sup>n</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 43 to 45.

The auxiliary amino acid sequence having a (Gly Ala Gln Gly Pro Ala Gly Pro Gly)<sup>o</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 46 to 48.

The auxiliary amino acid sequence having a (Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly Ala Pro Gly Leu Gln)<sup>p</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 49 to 51.

The auxiliary amino acid sequence having a (Gly Ala Pro Gly Thr Pro Gly Pro Gln Gly Leu Pro Gly Ser Pro)<sup>q</sup> sequence includes amino acid sequences represented by SEQ ID NOs: 52 to: 54.

Among these amino acid sequences, preferred are amino acid sequences represented by SEQ ID NO:9, 10, 12, 13, 14, 15, 16, 18, 19, 21, 22, 24, 25, 26, 27, 28, 30, 31, 32, 34, 35, 37, 38, 40, 41, 43, 44, 46, 47, 49, 50, 52, or 53, more preferably amino acid sequences represented by SEQ ID NO:10, 12, 13, 14, 16, 19, 22, 26, 27, 28, 29, 30, 32, 35, 38, 41, 44, 47, 50, or 53, and particularly preferably amino acid sequences represented by SEQ ID NO:12, 13, or 30.

The polypeptide (P) may include a branched chain, may be partially crosslinked, or may include a ring structure. However, preferably the polypeptide (P) is not crosslinked, more preferably is a linear-chain structure that is not crosslinked, and particularly preferably is a linear-chain structure that does not have a ring structure and is not crosslinked. The linear-chain structure includes a  $\beta$  structure (secondary structure where a linear peptide is folded, and the folded parts are lined up in parallel, between which hydrogen bonds are formed).

From the viewpoints of cell-adhering property, thermal resistance, etc., preferably the polypeptide (P) has a structure where a minimum amino acid sequence (X) and an auxiliary amino acid sequence (Y) are alternately chemically bonded. In this case, from the viewpoints of cell-adhering property etc., the number of repeated units (X-Y) of the minimum amino acid sequence (X) and the auxiliary amino acid sequence (Y) is preferably 1 to 50, more preferably 2 to 40, particularly preferably 3 to 30, and most preferably 4 to 20.

Moreover, the content numbers of minimum amino acid sequences (X) and auxiliary amino acid sequences (Y) may be the same or different. If they are different, preferably either one of the content numbers thereof is smaller than the other number by one (in this case, the number of the auxiliary amino acid sequences (Y) is preferably smaller). The content ratio (X/Y) of the number of minimum amino acid sequences (X) to the number of auxiliary amino acid sequences (Y) in the polypeptide (P) is preferably 0.66 to 1.5, more preferably 0.9 to 1.4, and particularly preferably 1 to 1.3.

Moreover, the terminal site of the polypeptide (P) (from the minimum amino acid sequence (X) or the auxiliary amino acid sequence (Y) to the peptide terminal) may contain another amino acid. If another amino acid is contained, the content thereof is preferably 1 to 1000 per polypeptide, more preferably 3 to 300, and particularly preferably 10 to 100.

The number-average molecular weight (Mn) of the polypeptide (P) is preferably 1,000 to 1,000,000, more preferably 2,000 to 700,000, particularly preferably 3,000 to 400,000, and most preferably 4,000 to 200,000. The number-average molecular weight (Mn) of the polypeptide may be obtained by a publicly known method such as a method of separating a measurement sample (such as polypeptide) by SDS-PAGE (SDS polyacrylamide gel

electrophoresis), and comparing the migration distance thereof with that of a reference material (hereunder the same).

Hereunder are preferred examples of the polypeptide (P).

(1) A case where the minimum amino acid sequence (X) is an Arg Gly Asp sequence (x1)

A polypeptide of about 110,000 Mn having a structure where 13 (x1) and 13 (Gly Ala Gly Ala Gly Ser)<sub>9</sub> sequences (13)(y1) are alternately chemically bonded ("ProNectin F", ProNectin: registered trademark (Japan and US), manufactured by Sanyo Chemical Industries. (hereunder the same));

A polypeptide of about 20,000 Mn having a structure where 5 (x1) and 5 (Gly Ala Gly Ala Gly Ser)<sub>3</sub> sequences (12)(y2) are alternately chemically bonded ("ProNectin F2");

A polypeptide of about 10,000 Mn having a structure where 3 (x1) and 3 (Gly Val Pro Gly Val)<sub>2</sub> Gly Gly (Gly Ala Gly Ala Gly Ser)<sub>3</sub> sequences (30)(y3) are alternately chemically bonded ("ProNectin F3"); and the like.

(2) A case where the minimum amino acid sequence (X) is a Ile Lys Val Ala Val sequence (x2)

"ProNectin L", "ProNectin L2", or "ProNectin L3" where the Arg Gly Asp sequence (x1) of ProNectin F, ProNectin F2, or ProNectin F3 is changed into a Ile Lys Val Ala Val sequence (7)(x2), and the like.

(3) A case where the minimum amino acid sequence (X) is a Tyr Ile Gly Ser Arg sequence (x3)

"ProNectin Y", "ProNectin Y2", or "ProNectin Y3" where the Arg Gly Asp sequence (x1) of ProNectin F, ProNectin F2, or ProNectin F3 is changed into a Tyr Ile Gly Ser Arg sequence (x3), and the like.

Moreover, in addition to the polypeptides of (1) to (3), there may be also preferably used RetroNectin (recombinant human fibronectin CH-296) manufactured by Takara (a polypeptide of about 60,000 Mn having an Arg Gly Asp sequence (x1) and a Leu Asp Val sequence as the minimum amino acid sequence (X)), and RGDS-Protein A manufactured by Takara (a polypeptide of about 30,000 Mn having an Arg Gly Asp sequence (x1) as the minimum amino acid sequence (X)), where these polypeptides do not contain an auxiliary amino acid sequence (Y). Therefore, the thermal resistance and the like thereof are inferior to those of the abovementioned (1) to (3). Moreover, the amino acid sequences of these polypeptides are disclosed in Japanese Unexamined Patent Publication No. H2-311498 (the contents of US5198423A are incorporated in the present application by reference).

The method of manufacturing the polypeptide (P) is not specifically limited, and it may be manufactured in the same manner as an already known method for synthesizing a peptide,

and it may be synthesized by for example an organic synthesis method (such as a solid phase synthesis method and a liquid phase synthesis method), a biochemical synthesis method [genetic recombinant bacteria (such as yeast, bacteria, and *E. coli*)], and the like. Regarding the organic synthesis method, there may be used a method described in for example "Lectures on Biochemical Experiments, Second Series, 2, Chemistry of Proteins Vol. 2" pages 641-694, edited by The Japanese Biochemical Society, (published by Tokyo Kagaku Dojin; 20 May 1987), and the like. Regarding the biochemical synthesis method, there may be used methods described in, for example, Published Japanese translation No. H3-502935 of PCT International Publication (the contents of US5243038A, US5496712A, US5514581A, US5606019A, US5641648A, US5723588A, US5770697A, US5773249A, US5808012A, US5830713A, US6018030A, US6140072A, US6184348B1, US6355776B1, US6380154B1, US2003083464A1, and US2003176355A1 are incorporated in the present application by reference), and the like. From the point of being able to readily synthesize the polypeptide (P) of a high molecular weight, preferred is a biochemical synthesis method using genetic recombinant bacteria, and particularly preferred is a synthesis method using genetic recombinant *E. coli*.

If the support contains the polypeptide (P), the (P) may be contained in the surface of the support, and the (P) is bonded to the surface of the support by a chemical bond (such as an ionic bond, a hydrogen bond, and/or a covalent bond) and/or a physical adsorption (adsorption by Van der Waals force). Among these, preferred is a chemical bond, and more preferred is a covalent bond.

The reaction for covalently bonding the polypeptide (P) to the support can be performed by a publicly known method. Examples thereof include methods described in "Fundamentals and Experiments of Peptide Synthesis" published by Maruzen (5 October 1997). More specifically these are as in (1) to (3) hereunder.

(1) In the case where a polypeptide having a primary amino group or a secondary amino group and a support not containing a polypeptide (P) (hereunder, P non-contained support) but having a carboxyl group are subject to reaction, the carboxyl group of the P non-contained support is previously reacted with a carbodiimide compound, so as to obtain acylisourea ( $R'-N=C(OCOR)-NH-R'$  (-OCOR is the site derived from the support)). Then, by adding the polypeptide having a primary amino group or a secondary amino group to this acylisourea, the P non-contained support and the polypeptide can be amide bonded.

The carbodiimide compound includes N,N'-dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and the like.



(2) In the case where a polypeptide having a primary amino group or a secondary amino group and a P non-contained support having a hydroxyl group are subject to reaction, the hydroxyl group of the P non-contained support is previously reacted with a carbonyldiimidazole compound, so as to obtain an imidazole derivative (R-Im, where Im denotes an imidazoline ring and R is derived from the support). Then, by adding the polypeptide having a primary amino group or a secondary amino group to this imidazole derivative, the P non-contained support and the polypeptide can be N-C bonded.

The carbonyldiimidazole compound includes N,N'-carbonyldiimidazole, and the like.

(3) In the case where a polypeptide having a hydroxyl group and a P non-contained support having a carboxyl group are subject to reaction, the carboxyl group of the P non-contained support is previously reacted with a carbodiimide compound, so as to obtain acylisourea. Then, by adding the polypeptide having a hydroxyl group to this acylisourea, the P non-contained support and the polypeptide can be ester bonded.

The methods of physically adsorbing, ionically bonding, and/or hydrogen-bonding a polypeptide to a P non-contained support include a production method of putting the polypeptide and the P non-contained support into a solvent or the like, and mixing them. The solvent is not specifically limited. However, there may be used water (such as tap water, ion exchanged water, distilled water, and ion exchanged distilled water) as well as an aqueous solution containing inorganic salt, organic acid salt, acid and/or base (for example, Japanese Unexamined Patent Publication No. 2003-189848), and the like. The contents (weight %) of inorganic salt, organic acid salt, acid and base is preferably 0.001 to 50 based on the weight of the aqueous solution, more preferably 0.005 to 30, and particularly preferably 0.01 to 10.

Among these solvents, preferred are water and aqueous solution containing inorganic salt, acid and/or base, more preferably ion exchanged distilled water and aqueous solution containing inorganic salt, acid and/or base, and particularly preferably aqueous solution containing inorganic salt, acid and/or base.

If the support contains the polypeptide (P), the content ( $\mu\text{g}/\text{cm}^2$ ) of the polypeptide (P) is preferably 0.0001 to 100000 per  $1\text{ cm}^2$  of the mean surface area of the support, more preferably 0.001 to 10000, and particularly preferably 0.01 to 1000. Within this range, the efficiency of cell culture is further improved.

The content of the polypeptide (P) can be obtained as follows.

(1) The content ( $\mu\text{g}/\text{g}$ ) of the polypeptide (P) per unit weight of the support is measured by for example an immunoassay (described in Japanese Unexamined Patent Publication No.

2004-049921 and the like). That is, the support and an antibody which is bindable to the polypeptide (P) and labeled with an enzyme (hereunder, enzyme labeled antibody 1) are reacted, then the enzyme level of the reacted enzyme labeled antibody 1 is measured, and thereby the content of the polypeptide (P) per unit weight is measured.

(2) Next, using a surface shape measuring microscope (3D shape measuring microscope utilizing the confocal principle, such as VK-9500 manufactured by Keyence Corporation), three dimensional data of the shape of the top surface (for example,  $20\mu\text{m} \times 20\mu\text{m}$ ) of the support fixed to a slide glass with an adhesive or the like (hereunder, fixed support), is obtained. Then, small pore (having a diameter of less than  $1\mu\text{m}$ ) parts are uniformly excluded from this three dimensional data (corrected as a flat surface) so as to obtain the partial surface area (A) of the support having ribs (ridges) and the like. Moreover, small pore (having a diameter of less than  $1\mu\text{m}$ ) parts, ribs (ridges), and the like are uniformly excluded from the three dimensional data of the surface shape (corrected as a flat surface) so as to obtain the partial surface area (B) of the support having a flat surface. Furthermore, the three dimensional data of 9 supports are measured in the same manner, to obtain the partial surface areas (A) of these supports and the partial surface areas (B) of these supports. For these 10 supports, the mean partial surface area (HA) of the partial surface areas (A) of the supports having ribs and the like, and the mean partial surface area (HB) of the partial surface areas (B) of the supports having flat surfaces are calculated.

If the support is globular, the mean surface area per unit weight of the support is calculated from an equation (mean surface area per unit weight ( $\text{cm}^2/\text{g}$ )= $[4 \times \pi \times (\text{ra}/2/10000)^2]/[4/3 \times \pi \times (\text{ra}/2/10000)^3 \times d] \times [(HA)/(HB)]$ ), where ra denotes a particle diameter of the support, and d denotes a true specific gravity of the support.

If the support is non-globular (rod, hexahedron, or plate shape) or porous, disengaging the calculation of the above method, the mean surface area per unit weight of the support is calculated as follows.

Using a specific surface area meter (for example, QUANTASORB manufactured by Yuasa Ionics Inc.), the surface area of the support is measured (measurement gas; He/Kr = 99.9/0.1 in volume ratio, detection gas; nitrogen). The mean surface area per unit weight ( $\text{cm}^2/\text{g}$ ) of the support is calculated from an equation (surface area of support/weight of support).

(3) The content of the polypeptide (P) per unit weight ( $\mu\text{g/g}$ ) is divided by the mean surface area per unit weight ( $\text{cm}^2/\text{g}$ ) to calculate the content per  $1\text{ cm}^2$  of the mean surface area of the support ( $\mu\text{g}/\text{cm}^2$ ).

The cell dispersing agent means a cell dispersing agent free from animal-origin components used for subculture, and used for the purpose of detaching cells from the support. The cell dispersing agent includes a chelating agent (such as EDTA), a serum-free medium at 2 to  $30^\circ\text{C}$ , a protease originated from a plant (such as papain), a protease originated from genetic recombinant bacteria (trypsin-like enzyme (such as rProtease manufactured by Invitrogen)), and the combination thereof. Among these, from the viewpoints of detachability of cells from the support etc., preferred are a protease originated from a plant, a protease originated from genetic recombinant bacteria, and the combination thereof, and more preferably a protease originated from genetic recombinant bacteria.

In the method of producing a virus of the present invention, a large amount of virus can be produced by: adhering adhesive cells to a support free from animal-origin components; culturing the adhesive cells in a medium free from animal-origin components; subculturing the cultured adhesive cells using a cell dispersing agent free from animal-origin components; and then inoculating and proliferating a virus in the cells obtained by culturing the adhesive cells.

The culture (hereunder, preculture) for obtaining the adhesive cells used for cell dissemination is not specifically limited, and there may be used any one of materials containing animal-origin components (such as a support, a medium, and a cell dispersing agent), and materials free from animal-origin components (such as a support, a medium, and a cell dispersing agent). However, in the preculture, there are preferably used only materials free from animal-origin components (such as a support, a medium, and a cell dispersing agent).

The method of adhering the adhesive cells obtained by the preculture to the support may be a normal method, and there may be applied a method of disseminating the cells in a medium or a support, and the like.

The dissemination amount of the adhesive cells ( $10^4/\text{cm}^2$ ) is determined according to the type of the adhesive cell. However, preferred is 0.001 to 1000 per  $1\text{ cm}^2$  of the mean surface area of the support, more preferably 0.01 to 100, and particularly preferably 0.1 to 10. The method of measuring the number of the adhesive cells is not specifically limited, however for example the number of cell nuclei may be measured by a cell nucleus counting method using a crystal violet by Wezel. Moreover, the cell density in a medium ( $10^4/\text{mL}$ ) is preferably 0.01 to

10000 per 1mL of medium, more preferably 0.1 to 1000, particularly preferably 1 to 100, even more particularly preferably 5 to 50, and most preferably 10 to 30.

The incubation period (day) for the adhesive cells is preferably 3 to 30, more preferably 4 to 21, particularly preferably 5 to 15, still more preferably 6 to 10, and most preferably 7 or 8. Although it varies depending on the type of the adhesive cell, in the case of an 8 days culture, the cell density in a medium increases about 3 to 30 times compared to that at the time of starting the culture.

From 1/3 to all of the amount of the medium is preferably exchanged every 1 to 5 days.

The density of carbon dioxide during the incubation (volume %) is preferably 2 to 10 based on the volume of the incubation atmosphere, more preferably 4 to 6, and particularly preferably 5.

The incubation temperature (°C) is preferably 25 to 42, more preferably 30 to 40, particularly preferably 35 to 39, and most preferably 37.

If a microcarrier (particle diameter; 20 to 500  $\mu\text{m}$ , surface area; 100cm<sup>2</sup> to 100m<sup>2</sup>, or the like) is used as the support, there may be used (1) a spinner flask or a vessel, (2) a radial flow type reactor, etc., as a culture vessel.

(1) If a spinner flask, a vessel, or the like is used, there may be applied, for example, a method of putting adhesive cells, a microcarrier, and a medium into the culture vessel (volume; 100mL to 100L or the like), and culturing while stirring.

(2) If a radial flow type reactor or the like is used, there may be applied, for example, a method of setting a microcarrier in the culture vessel (total volume; 100mL to 100L or the like), then culturing while circulating the medium containing the adhesive cells.

On the other hand, if a hollow fiber (inner diameter: 10 to 500  $\mu\text{m}$  or the like) is used as the support, there may be applied, for example, a method of adding a medium containing the adhesive cells into a cartridge (total capacity; 10mL to 10L or the like), then culturing while circulating the medium in the hollow fiber.

Moreover, if a roller bottle (total capacity; 0.1 to 20L or the like) is used as the support, there may be applied, for example, a method of adding a medium containing the adhesive cells into the culture vessel, then culturing while stirring.

The subculture using a cell dispersing agent means to make the adhesive cells obtained from the culture in the above manner, into a cell dispersion by the cell dispersing agent, and then performing the culture of the adhesive cells again using this cell dispersion.



The timing for inoculating the virus in the cells obtained by culturing the adhesive cells is preferably between the 3rd to 30th day from the start of culturing the adhesive cells, more preferably between the 4th to 21st day, particularly preferably between the 5th to 15th day, still more preferably between the 6th to 10th day, and most preferably between the 7th and 8th day.

Moreover, the cell density ( $10^4/\text{mL}$ ) of the cultured cells for inoculating the virus is preferably 0.2 to 200000, more preferably 2 to 20000, particularly preferably 20 to 2000, even more particularly preferably 100 to 1000, and most preferably 200 to 500.

The medium is preferably exchanged with a serum-free medium prior to the inoculation of the virus. More preferably, the medium of the cell culture is removed, and PBS (0.02M phosphate buffer solution or the like) and/or a serum-free medium are added, which is stirred for 1 minute to 1 hour. Then, the added PBS and/or serum-free medium are removed, and the serum-free medium is added.

This serum-free medium is preferably the same one that has been used for the cell culture.

This serum-free medium may or may not contain a cell dispersing agent. In the case of an Influenza virus, preferably the serum-free medium contains a cell dispersing agent. If the serum-free medium contains a cell dispersing agent, the content thereof (volume %) is preferably 0.5 to 40 based on the volume of the serum-free medium, more preferably 1 to 20, and particularly preferably 2 to 10.

The M.O.I (multiplicity of infection) for inoculating the virus varies depending on the types of cell and virus, however preferred is 1 to 0.0000001, more preferred is 0.1 to 0.00001, and particularly preferred is 0.05 to 0.0001.

The incubation period (growth period; day) of the virus is preferably 2 to 14, and more preferably 3 to 10.

The incubation temperature (growth temperature; °C) of the virus is preferably 30 to 39, more preferably 32 to 38, and particularly preferably 33 to 37.

The pH of the medium is preferably controlled within a fixed range, and the range is preferably 6 to 9, more preferably 6.5 to 8.5, and particularly preferably 7 to 8.

The vaccine that can be produced using the method of producing a virus of the present invention is not specifically limited. However, preferred are a Japanese encephalitis vaccine, a Dengue fever vaccine, a West Nile fever vaccine, an Influenza vaccine, a Rabies vaccine, a Varicella vaccine, a Polio vaccine, a Hepatitis A vaccine, a Measles vaccine, a Rubella vaccine, and a Mumps vaccine, more preferred are a Japanese encephalitis vaccine, a Dengue fever

vaccine, a West Nile fever vaccine, an Influenza vaccine, a Polio vaccine, a Measles vaccine, a Rubella vaccine, and a Mumps vaccine, particularly preferred are an Influenza vaccine, a Measles vaccine, a Rubella vaccine, and a Mumps vaccine, and most preferred is an Influenza vaccine.

According to the present invention, since culture materials free from animal-origin components are used in the process of cell culture and virus production, a large amount of adhesive cells which are safe and stable quality, can be cultured. Furthermore, it has advantages in that it can minimize the likelihood of contamination due to foreign substances, it is free from unknown infectious agents, and thus there is no need of performing a treatment for deactivating the infectious agent.

The present invention is not limited to the above contents of description, and various modification can be made within the scope of the present invention.

Hereunder is a detailed description of the present invention, with reference to Examples and drawings. However, the present invention is not limited to these Examples.

<Culture for obtaining adhesive cell used for cell dissemination>

Vero during preculture in Dulbecco's minimum essential medium (DMEM) medium + 5 volume % fetal bovine serum (FBS) (manufactured by Invitrogen) were dispersed by a diluted solution having a cell dispersing agent (rProtease (registered trademark) manufactured by Invitrogen) diluted with PBS as required, under a condition of 37°C, and the filtrate was removed by centrifugation, to obtain the Vero (S) for cell dissemination.

#### Example 1

<Production of Japanese encephalitis virus using serum-free medium and ProNectin F-bonded nylon beads>

(1) ProNectin F was diluted with phosphate buffer solution (PBS) so that the density of ProNectin F became 300 $\mu$ g/ml, to produce ProNectin F solution. 100ml of water-soluble carbodiimide solution (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (300mM aqueous solution manufactured by Dozind) was added with 50g of 12 nylon beads (particle diameter 150 to 250 $\mu$ m, manufactured by Trial Corporation), and stirred by a stirrer for 2 hours. Then, 100ml of phosphate buffer solution (pH=7.2) was added thereto and the solution was removed by means of suction. This operation was performed 5 times (washing), and thereby carbodiimide bonded nylon beads (1) were obtained. Next, these carbodiimide bonded nylon beads (1) were soaked in 100ml of ProNectin F solution, and stirred for 2 hours. Then, 100ml of

phosphate buffer solution (pH=7.2) was added thereto, and the solution was removed by means of suction. This operation was performed 3 times (washing), and then it was stirred in ammonia aqueous solution (300mM, 100ml) for 2 hours. Then, 100ml of ion exchanged water was added, and the solution was removed by means of suction. This operation was performed for 3 times (washing). Next, it was dried by blowing hot wind of 100°C for 60 minutes, and ProNectin F-bonded nylon beads (ProNectin F bonding amount: 0.3μg/cm<sup>2</sup>) were obtained.

(2) A sample 4 comprising a serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components and ProNectin F-bonded nylon beads free from animal-origin components was put into a spinner flask (F7689 manufactured by Techne Incorporated).

Next, the Vero (S) for cell dissemination were disseminated in the spinner flask containing the sample 4 so that the cell density became  $2 \times 10^5$  cells/mL, so as to make the operation volume (M) 100mL. Then, the culture solution containing the cells, the medium, and the microcarrier was stirred at 25 rpm so as to adhere the cells to the microcarrier. Then, the cells were cultured using a magnetic stirrer (MCS-104L manufactured by Techne Incorporated) at a stirring rotational speed of 25 rpm to 35 rpm in a thermostatic chamber at 37°C for 24 days.

The subculture of the cells was performed every 8 days. From the 3rd day of the cell dissemination, 50 volume % of medium was exchanged every day. The cells were dispersed by a diluted solution having a cell dispersing agent free from animal-origin components (rProtease (registered trademark) manufactured by Invitrogen) 5-fold diluted with PBS, under a condition of 37°C, and the filtrate was removed by centrifugation. Then, the required amount thereof was disseminated in a spinner flask that had been previously prepared, so as to perform the subculture operation.

The change in the cell density of the sample 4 with time was obtained by a general method such as hematometry and microscopy. A graph showing the cell density (cells/mL) on the y-axis and time on the x-axis, is shown in FIG. 1.

On the 24th day from the start of the cell culture, the culture solution in the spinner flask was removed, and washing with PBS was performed twice. Then, Japanese encephalitis virus (JEV) was inoculated at M.O.I=0.01, and cultured at a stirring rotational speed of 35 rpm in a thermostatic chamber at 37°C. On the 2nd day of the JEV culture, 1.5mL of 7.5 volume % sodium hydrogencarbonate solution was added into each spinner flask, so as to make pH 7.2.

Sampling was performed between the 2nd to 8th day of the JEV culture, and the number of cells was measured by measuring the number of nuclei after citric acid treatment. Regarding the index of JEV growth, (1) HA (Hemagglutination) value was measured according to the usual method, and (2) in ELISA (Enzyme-linked immunosorbent assay), antibody purification of anti-JEV antiserum was performed by means of protein A column and the reaction specificity was confirmed by means of Western Blotting, and then the purified antibodies were labeled with peroxidase, so as to construct a sandwich ELISA. Regarding the ELISA value, the autologous value from reference Japanese encephalitis vaccine Beijing strain Lot.197-P was added, and the purified inactive JEV solution was used as a reference antigen. A graph showing the change in the JEV production quantity with time by ELISA, is shown in FIG. 2. A graph showing the change in the JEV production quantity with time by HA, is shown in FIG. 3. Moreover, the highest values of ELISA and HA measurement values are shown in Table 1.

Table 1

	Cell number at time of virus inoculation ( $\times 10^6$ cells/mL)	HA	ELISA (U/mL)
Sample 1	1.6	256	1949
Sample 2	3	128	1087
Sample 3	2.4	64	626
Sample 4	3	512	2367

#### Comparative Example 1

<Production of Japanese encephalitis virus using serum medium and dextran beads (product name: Cytodex1 manufactured by Amersham)>

The virus was produced in the same manner as that of Example 1, except that a sample 1 {serum medium (DMEM medium + 5 volume % FBS), and dextran beads free from animal-origin components} was used instead of the sample 4 that has been described in Example 1.

#### Comparative Example 2

<Production of Japanese encephalitis virus using serum-free medium and dextran beads (product name: Cytodex1 manufactured by Amersham)>

The virus was produced in the same manner as that of Example 1, except that a sample 2 {serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin



components, and dextran beads free from animal-origin components} was used instead of the sample 4 that has been described in Example 1.

### Comparative Example 3

<Production of Japanese encephalitis virus using serum-free medium and dextran beads coated with denatured pig collagen (product name: Cytodex3 manufactured by Amersham)>

The virus was produced in the same manner as that of Example 1, except that a sample 3 {serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components, and dextran beads coated with denatured pig collagen} was used instead of the sample 4 that has been described in Example 1.

For Comparative Examples 1 to 3, in the same manner as that of Example 1, the number of cells at the time of virus inoculation, and the highest values of ELISA and HA measurement values are shown in Table 1. A cell density-time graph is shown in FIG. 1. An ELISA Titer-day graph is shown in FIG. 2. A HA Titer-day graph is shown in FIG. 3.

From FIG. 1 and Table 1, it can be seen that the cell density of Example 1 (sample 4) was the highest on the 8th, 16th, and 24th day from the start of the culture, and the cells of Example 1 (sample 4) were stably and efficiently grown compared to Comparative Example 1 (sample 1), Comparative Example 2 (sample 2), and Comparative Example 3 (sample 3).

Moreover, from FIG. 2, FIG. 3, and Table 1, it can be seen that Example 1 (sample 4) showed higher values of both ELISA and HA compared to Comparative Example 1 (sample 1), Comparative Example 2 (sample 2), and Comparative Example 3 (sample 3), and Japanese encephalitis virus were efficiently grown.

### Example 2

<Production of influenza virus using serum-free medium and ProNectin F-bonded nylon beads>

A sample 8 comprising a VP-SFM medium and ProNectin F-bonded nylon beads was put into a spinner flask (F7689 manufactured by Techne Incorporated).

Next, the Vero (S) for cell dissemination were disseminated in the spinner flask containing the sample 8 so that the cell density became  $2 \times 10^5$  cells/mL, so as to make the operation volume (M) 100mL. Then, the culture solution containing the cells, the medium, and the microcarrier was stirred at 35 rpm so as to adhere the cells to the microcarrier. Then, the cells were cultured using a magnetic stirrer (MODEL1104M manufactured by Wakenyaku Co. Ltd.) at a stirring rotational speed of 35 rpm in a thermostatic chamber at 37°C for 24 days.

The subculture of the cells was performed every 8 days. From the 3rd day of the cell dissemination, 50 volume % of medium was exchanged every day. The cells were dispersed by rProtease that had been used for the cell subculture, under a condition of 37°C, and the filtrate was removed by centrifugation. Then, the required amount thereof was disseminated in a spinner flask that had been previously prepared, so as to perform the subculture operation.

Regarding the cell washing process, on the 24th day from the start of the cell culture, the stirring of the spinner flask was stopped, the culture solution was removed therefrom, 50mL of PBS was added therein, and the flask was stirred in a thermostatic chamber at 33°C for 20 minutes. Next, the cell washing operation was performed in the same condition, but with a VP-SFM medium. Then, the VP-SFM medium was removed, and another VP-SFM medium was newly added into the spinner flask to make the volume 95mL. 5mL of rProtease was added into this spinner flask and influenza virus (B/Johannesburg/5/99) was inoculated at M.O.I=0.001. The influenza virus was cultured at a stirring rotational speed of 35 rpm in a thermostatic chamber at 33°C, while the cap of the spinner flask was loosened. After the influenza virus was inoculated, the pH of the medium was kept at pH7.2 to pH7.8 with a 7.5 volume % sodium hydrogencarbonate solution (0.4mL of 7.5 volume % sodium hydrogencarbonate solution was added after 18 hours of the influenza virus culture, and 0.6mL thereof was added after 42 hours in the same manner). Sampling was performed after 67 hours of the influenza virus culture. The HA method using avian red blood corpuscle was used as an index of the influenza virus growth, and the HA value was 128 times.

### Example 3

<Production of influenza virus using serum-free medium and ProNectin F2-bonded nylon beads>

The virus was produced in the same manner as that of Example 2, except that ProNectin F2-bonded nylon beads were used instead of the ProNectin F-bonded nylon beads that have been described in Example 2 (ProNectin F2 bonding amount:  $0.3\mu\text{g}/\text{cm}^2$ , the beads were produced in the same manner as that of the ProNectin F-bonded nylon beads, except that ProNectin F2 was used instead of ProNectin F.) The HA value obtained in the same matter as that of Example 2 was 128 times.

### Example 4

<Production of influenza virus using serum-free medium and ProNectin F3-bonded nylon beads>

The virus was produced in the same manner as that of Example 2, except that ProNectin F3-bonded nylon beads were used instead of the ProNectin F-bonded nylon beads that have been described in Example 2 (ProNectin F3 bonding amount:  $0.2\mu\text{g}/\text{cm}^2$ , the beads were produced in the same manner as that of the ProNectin F-bonded nylon beads, except that ProNectin F3 was used instead of ProNectin F.) The HA value obtained in the same matter as that of Example 2 was 128 times.

#### Comparative Example 4

<Production of influenza virus using serum medium and dextran beads (product name: Cytodex1 manufactured by Amersham)>

The virus was produced in the same manner as that of Example 2, except that a sample 5 {serum medium (DMEM medium + 5 volume % FBS), and dextran beads free from animal-origin components} was used instead of the sample 8 that has been described in Example 2. The HA value obtained in the same matter as that of Example 2 was 32 times.

#### Comparative Example 5

<Production of influenza virus using serum-free medium and dextran beads (product name: Cytodex1 manufactured by Amersham)>

The virus was produced in the same manner as that of Example 2, except that a sample 6 {serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components, and dextran beads free from animal-origin components} was used instead of the sample 8 that has been described in Example 2. The HA value obtained in the same matter as that of Example 2 was 4 times.

#### Comparative Example 6

<Production of influenza virus using serum-free medium and dextran beads coated with denatured pig collagen (product name: Cytodex3 manufactured by Amersham)>

The virus was produced in the same manner as that of Example 2, except that a sample 7 {serum-free medium (VP-SFM manufactured by Invitrogen) free from animal-origin components, and dextran beads coated with denatured pig collagen} was used instead of the sample 8 that has been described in Example 2. The HA value obtained in the same matter as that of Example 2 was 4 times.

HA values were much higher in Example 2, Example 3, and Example 4 compared to Comparative Example 4, Comparative Example 5, and Comparative Example 6, and it can be seen that the influenza virus was very effectively grown.

[Sequence Table]